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The Role of D2–40, and Podoplanin in Differentiating Mesotheliomas from Primary Adenocarcinomas of the Lung and Metastatic Carcinomas of the Pleura

D2-40 ve Podoplanin'in Mezotelyomaların Akciğerin Primer Adenokarsinomları ve Plevranın Metastatik Karsinomlarından Ayrımındaki Rolü

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ABSTRACT

Objective: The aim of this study was to assess the diagnostic and differential significance of two recently introduced positive mesothelial markers, namely podoplanin and D2-40.

Material and Method: The study group included a total of 55 cases: 11 cases of malignant mesothelioma and equal number of cases (n:11) of primary lung adenocarcinoma, bronchioloalveolar carcinoma, metastatic carcinoma of the pleura and mesothelial proliferation. D2-40, podoplanin, calretinin, cytokeratin 5/6, carcinoembryonic antigen, epithelial specific antigen (MOC-31) were immunohistochemically studied in all cases. The extent of staining was graded between 1+ and 4+.

Results: While the 7 (63.6%) of the mesotheliomas and 10 of the mesothelial proliferations (90.9%) stained positive with D2-40, no positive staining was noted in primary lung adenocarcinoma, bronchioloalveolar carcinoma, metastatic carcinoma of the pleura. 9 of the mesotheliomas (81.8%) and 10 of the mesothelial proliferations (90.9%) were positive with podoplanin whereas no positive staining was observed in the other groups. Eight of the primary lung adenocarcinomas (72.7%), seven of the bronchioloalveolar carcinomas (63.6%), and eight of the metastatic carcinomas of the pleura (72.7%) were positive for MOC-31 while only one of the mesotheliomas (9%) was positive and no mesothelial proliferation cases were positive. The specificity of podoplanin, D2-40, calretinin and cytokeratin 5/6 for mesothelioma was 100% and their sensitivity was 81.8%, 63.6%, 81.8% and 54.5%, respectively. The highest sensitivity, (i.e. 100% sensitivity) for mesothelioma was observed with the D2-40, podoplanin, and calretinin combination.

Conclusion: D2-40 and Podoplanin were effective in differentiating malignant mesothelioma from primary lung adenocarcinomas, bronchioloalveolar carcinoma and metastatic carcinomas of the pleura. However, since they may not yield precise results it was therefore concluded that a panel combined with positive and negative mesothelial markers including these two markers will lead to more accurate results.

Key Words: Mesothelioma, Lung neoplasms, Adenocarcinoma, Metastasis, D2-40, Podoplanin, MOC-31

ÖZ

Amaç: En son pozitif mezotelyal belirleyici olarak tanımlanan podoplanin ve D2-40'ın malign mezotelyoma ile primer akciğer adenokarsinomu ve plevraya metastaz yapan adenokarsinomların ayırımında taşıdığı değeri araştırmak.

Gereç ve Yöntem: Ondokuz Mayıs Üniversitesi Tıp Fakültesi Patoloji Anabilim Dalı'nda 1988-2008 yılları arasında malign mezotelyoma tanısı almış 11 olgu ve buna eş sayıda primer akciğer adenokarsinomu, bronkioloalveoler karsinom, plevranın metastatik karsinomları ve mezotelyal proliferasyon tanısı almış toplam 55 olguya D2-40, podoplanin, kalretinin, sitokeratin 5/6, karsinoembriyonik antijen, epitelyal spesifik antijen (MOC- 31) ile immünhistokimyasal çalışma yapıldı. Boyanma yaygınlığı 1+ ile 4+ arasında derecelendirildi.

Bulgular: D2-40 ile 11 mezotelyoma olgusunun 7'si (%63,6), mezotelyal proliferasyon olgusunun 10'u (%90,9) pozitif boyanırken primer akciğer adenokarsinomu, bronkioloalveoler karsinom ve plevranın metastatik karsinomu olgularında pozitiflik görülmedi. Podoplanin ile mezotelyoma olgularının 9'u (%81,8), mezotelyal proliferasyon olgularının 10'u (%90,9) pozitif boyanırken diğer gruplarda pozitiflik görülmedi. Primer akciğer adenokarsinomlarının sekizi (%72.7), bronkioloalveoler karsinomların yedisi (%63.6), plevranın metastatik karsinomlarının sekizi (%72.7), MOC-31 ile reaksiyon verirken, mezotelyoma olgularının sadece birinde pozitif boyanma görüldü; mezotelyal proliferasyon olgularının hiçbirinde reaksiyon izlenmedi. Mezotelyoma için podoplanin, D2-40 ve kalretinin ve sitokeratin 5/6'nın özgüllükleri %100 bulundu. Duyarlılıkları ise sırasıyla %,81,8, %63,6, %81,8, %54,5 idi. Mezotelyoma için en yüksek yani % 100 duyarlılık, D2-40, podoplanin ve kalretinin kombinasyonu kullanıldığında saptandı.

Sonuç: D2-40 ve Podoplanin'in malign mezotelyoma ile primer akciğer adenokarsinomları, bronkioloalveoler karsinom ve plevranın metastatik karsinomlarının ayırımında etkin olduğu, ancak kesin sonuçlar vermediği; bu nedenle bu belirleyicilerin de içinde yer aldığı pozitif ve negatif mezotelyal belirleyiciler ile bir panel oluşturulmasının daha doğru sonuçlara ulaştırabileceği kanısına varıldı.

Anahtar Sözcükler: Mezotelyoma, Akciğer neoplazmları, Adenokarsinom, Metastaz, D2-40, Podoplanin, MOC-31

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INTRODUCTION

Malignant mesothelioma, first defined by Wagner in 1960, is a primary tumor that develops from mesothelial cells on the serosal surfaces, especially on the pleura (1). Mesotheliomas can display various cytomorphological and histological types. It is difficult to differentiate them, both macroscopically and microscopically, from primary lung adenocarcinomas and metastatic carcinomas involving pleura. Histochemical, immunohistochemical and electron microscopic methods are employed when clinical, radiological and histomorphological findings are not adequate for differential diagnosis (2).

Today, there is no clearly defined marker for mesothelioma (3). The immunohistochemical diagnosis of mesothelioma is generally possible with the panels composed of markers often synthesized in mesotheliomas (positive mesothelioma markers) and markers widely synthesized in carcinomas (negative mesothelioma markers) (3-5). In the present study, we combined positive mesothelial markers, calretinin and cytokeratin (CK) 5/6 and negative mesothelial markers, carcinoembryonic antigen (CEA) and MOC-31, with recently defined positive mesothelioma markers, D2-40, and podoplanin, and aimed to investigate their diagnostic value in the differentiation of malignant mesothelioma from primary lung adenocarcinomas and metastatic carcinomas of the pleura.

MATERIAL and METHOD

55 cases from Ondokuz Mayıs University Medical School, Department of Pathology obtained between 1988 and 2008 were included in the study. Patients' diagnoses belonged to one of the following 5 groups with 11 patients in each: Malignant mesothelioma, primary lung adenocarcinoma, bronchioloalveolar carcinoma, metastatic carcinoma of the pleura and mesothelial proliferation. Paraffin sections of the cases were re-evaluated and 4-micrometer sections were prepared from the appropriate paraffin blocks that represented the tumor best. The immunohistochemical study was performed using the streptavidin biotin peroxidase method with the primary antibodies D2-40 (monoclonal mouse, clone D2-40, DAKO, Carpinteria, USA), podoplanin (18H5, sc-59347, Santa Cruz, California, USA), CEA (monoclonal mouse, clone CD66e Ab-3, Thermo Scientific, CA, USA), epithelial specific antigen Ab-7 (monoclonal mouse, clone MOC 31, Thermo Scientific, CA, USA), calretinin (rabbit, clone SP13, Thermo Scientific, CA, USA), and cytokeratin 5/6 Ab-2 (monoclonal mouse, clone D5/16 B4, Labvision, CA, USA). The epithelial cells in thymus for D2-40, germinal central lymphocytes in tonsilla pallatina for CEA and MOC 31, colonic adenocarcinoma for calretinin and CK 5/6, pleural tissue sections for podoplanin were used as positive controls. MOC 31, CEA and calretinin were ready-for-use antibodies. Podoplanin, D2-40 and CK 5/6 were diluted at the rates of 1/50, 1/100 and 1/20 respectively. Endogenous peroxidase activities of the deparaffinized sections were blocked by incubating in 3% hydrogen peroxide solution for 10 minutes. The sections were boiled in citrate buffer for 35 minutes and left to cool before studying with antibodies except CK 5/6. For CK 5/6 the sections were incubated in trypsin for 15 minutes. Sections were processed with the primary antibodies MOC 31, CEA, calretinin and CK 5/6 for 1 hour, D2-40 for 2 hours and podoplanin for 18 hours. They were then incubated with biotin-added anti-immunoglobulin and streptavidin-peroxidase conjugate for 10 minutes. A kit (DAKO, Carpinteria, USA) including 3.3'-diamino benzidine (DAB) was used as the staining agent. Finally, the sections were stained with Mayer's hematoxyline for 60 seconds. All sections were washed with pH 7.6 phosphate buffer in every step up to the DAB application and washed with distilled water after the DAB. All procedures were performed at room temperature.

The results were evaluated under the Leica HMLB45 (Leica, Germany, 2000) light microscope. Cell membrane staining for D2-40, podoplanin and MOC 31, cytoplasmic and luminal membrane staining for CEA and cytoplasmic staining for calretinin and CK 5/6 were accepted as positive. The evaluation of the staining extent was made semiquantitatively and scored according to the following staining criteria (% of stained cells)=0%, negative; 1+ (1-25%); 2+ (26-50%); 3+ (51-75%); and 4+ (76-100%).

Statistical evaluation of the results was performed with the software "SPSS 12.,0 for Windows". Mann-Whitney-U and Wilcoxon Signed Ranks tests were used for the statistical evaluation and $p<0.05$ was considered significant. The sensitivity and specificity in differentiating malignant mesothelioma from primary lung adenocarcinomas was assessed for each mesothelial marker.

RESULTS

D2-40: Seven of the 11 (63.6%) malignant mesothelioma cases were positive for D2-40. Of these, staining was 4+ in 2, 3+ in 1, 2+ in 1 and 1+ in 3 of the cases. The staining was mostly continuous and apical along the cytoplasmic membranes, while intracytoplasmic staining was rarely noted (Figure 1A-D). No positive staining was observed in other malignancy groups. Ten (90.9%) of the mesothelial proliferation cases were positive. Of these, staining was

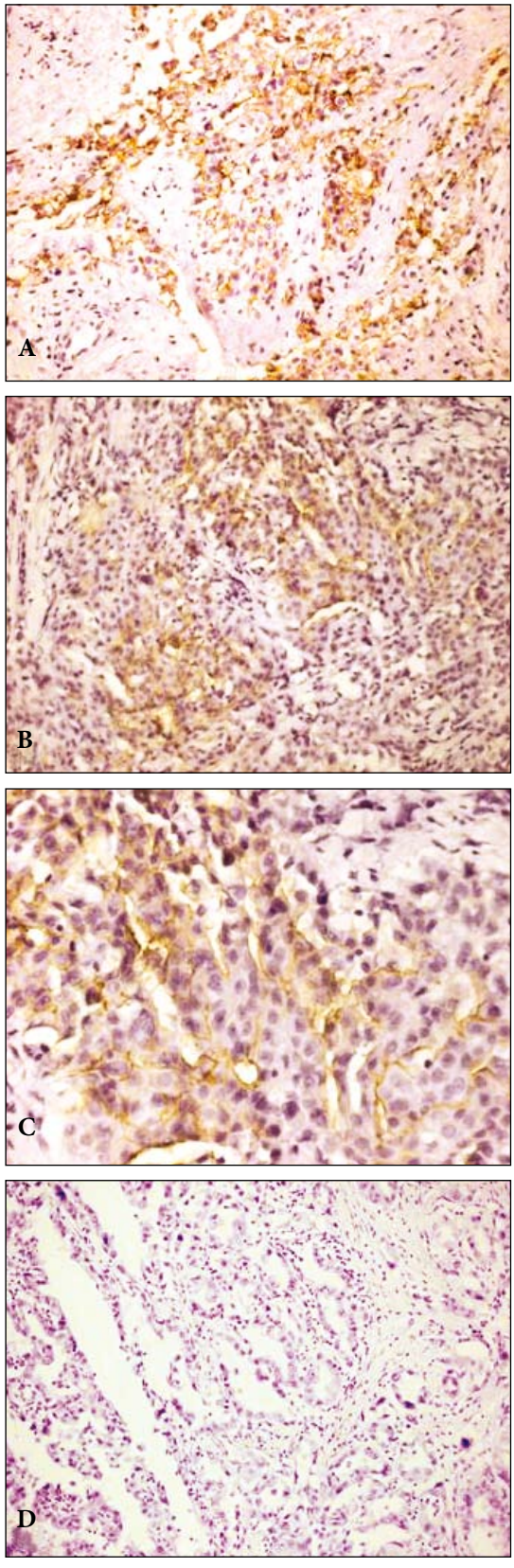

Figure 1: D2-40 expression in malignant mesothelioma (**A,B,** x200; **C,** x400). D2-40 was negative in primary lung adenocarcinoma (**D,** x200).

scored 4+ in 3, 3+ in 2 and 2+ in 5 of the cases. The staining pattern was similar to that of mesothelioma. When the D2-40 positiveness in mesothelioma cases was compared with those of primary lung adenocarcinomas, bronchioloalveolar carcinoma and metastatic carcinomas of the pleura the difference was found to be significant ($p<0.05$) (Table I). No significant difference was observed when compared with mesothelial proliferations ($p>0.05$).

Podoplanin: Nine of the 11 (81.8%) mesothelioma cases were positive for podoplanin. Scoring was 4+ in 1, 3+ in 5, 2+ in 1 and 1+ in 2 of the cases. The staining was continuous and apical membranous along the cytoplasmic membranes, with rare cytoplasmic and nuclear staining (Figure 2A-D). No positiveness was observed in other malignancy groups. Ten (90.0%) of the mesothelial proliferation cases of the podoplanin group were positive. Of these, staining was 4+ in 3, 3+ in 2 and 2+ in 5 of the cases. The staining pattern was similar to that of mesothelioma. When the podoplanin staining in malignant mesothelioma cases was statistically compared with those of primary lung adenocarcinomas, bronchioloalveolar carcinoma and metastatic carcinomas of the pleura the difference was significant ($p<0.05$) (Table I). There was no statistically significant difference between mesothelial proliferations and mesotheliomas ($p>0.05$).

Calretinin: Nine of the 11 (81.8%) mesothelioma cases were positive for calretinin. The staining was scored 4+ in 5, 3+ in 1 and 1+ in 3 of the cases. Both nuclear and cytoplasmic staining was observed in all cases. No positiveness was observed in other malignancy groups. Ten (90.9%) of the mesothelial proliferation cases were positive. The staining was 4+ in 1, 3+ in 4, 2+ in 4 and 1+ in 1 of the cases. The pattern of the staining was similar to that of mesothelioma. When statistically compared with those of primary lung adenocarcinomas, bronchioloalveolar carcinoma and metastatic carcinomas of the pleura the difference was significant ($p<0.05$). There was no statistically significant difference between the mesothelial proliferations and mesotheliomas ($p>0.05$).

CK 5/6: Six of the 11(54.5%) mesothelioma cases were positive for CK 5/6. Of these, staining was 3+ in 2 and 1+ in 4 of the cases. Cytoplasmic staining was observed in all cases. No positiveness was observed in other malignancy groups. Of the mesothelial proliferation cases, 9 (81.8%) were positive. Staining was scored 2+ in 1 and 1+ in 8 of the cases. Staining characteristic was similar to that of mesothelioma. When malignant mesothelioma cases were compared with primary lung adenocarcinomas, bronchioloalveolar carcinoma and metastatic carcinomas of the pleura the difference was statistically significant ($p<0.05$) (Table I).

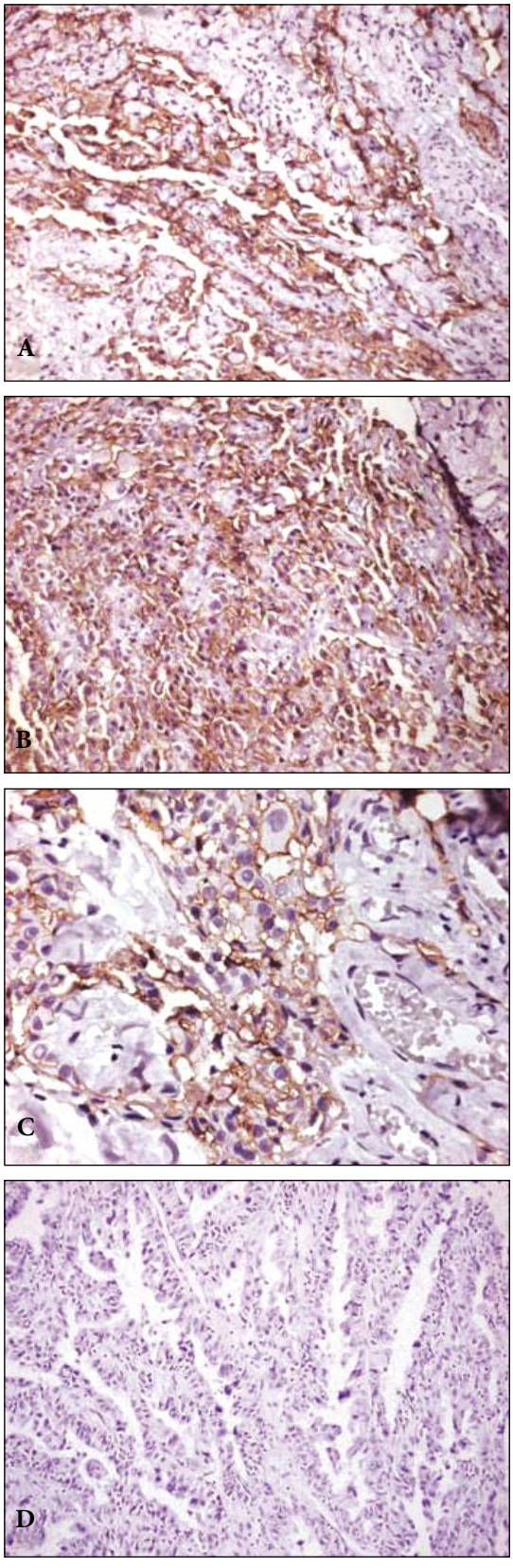

Figure 2: Podoplanin expression in malignant mesothelioma (**A,** x100; **B,** x200; **C,** x400). Podoplanin negativity in primary lung adenocarcinoma (**D,** x200).

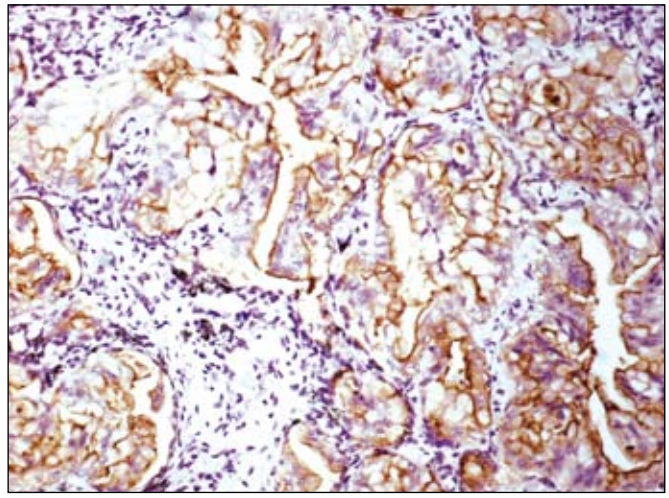

Figure 3: CEA expression in primary lung adenocarcinoma (CEA, x400).

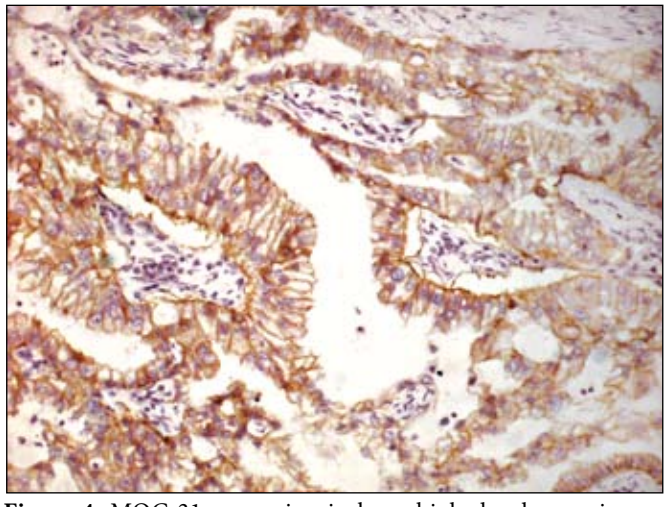

Figure 4: MOC-31 expression in bronchioloalveolar carcinoma (MOC 31, x400).

When mesotheliomas and mesothelial proliferations were compared for D2-40, podoplanin, calretinin and CK 5/6, no significant difference was observed ($p>0.05$).

CEA: Ten (90.9%) of the primary lung adenocarcinoma cases were positive for CEA. Staining was scored 4+ in 5, 3+ in 3 and 2+ in 2 of the cases. Mostly, cytoplasmic and luminal membrane staining was observed (Figure 3). Nine (81.8%) of the bronchioloalveolar carcinoma cases were positive. In 4 of the cases, staining was 4+, 2+ in 2 cases, and 1+ in 3 cases. Staining characteristic was similar to that of adenocarcinoma. Eight (72.7%) of the metastatic carcinomas of the pleura were positive. Staining was scored 4+ in 4, 3+ in 1, 2+ in 1 and 1+ in 2 of the cases. One (9%) of the mesothelioma cases was positive and its staining score was 2+.

Table I: p values obtained by the comparison of mesothelioma and primary lung adenocarcinomas for D2-40, podoplanin, calretinin and CK 5/6 expression scores

	D2-40	Podoplanin	Calretinin	CK 5/6
p value	0.002	<0.00001	<0.00001	0.006

Table II: p values obtained when the staining percentages of D2-40 and podoplanin in differentiating malignant mesothelioma from primary lung adenocarcinoma compared with the percentages of other positive mesothelial markers

Mesothelioma	Podoplanin vs. D2-40	Podoplanin vs. Calretinin	Podoplanin vs. CK 5/6	D2-40 vs. Calretinin	D2-40 vs. CK 5/6
p value	0.214	0.609	0.033	0.068	0.336

MOC-31: Eight (72.7%) of the primary lung adenocarcinoma cases were positive for MOC-31. Staining scores were 4+ in 3, 3+ in 1, 2+ in 2 and 1+ in 2 of the cases. Staining was observed mostly along the cell membrane. Seven (63.6%) of the bronchioloalveolar carcinoma cases were positive. Staining scores were 4+ in 1, 2+ in 1 and 1+ in 5 (Figure 4). Eight (72.7%) of the metastatic carcinomas of the pleura were positive. Staining was scored 4+ in 2, 3+ in 3, 2+ in 2 and 1+ in 1 of the cases. The staining characteristic was similar to that of adenocarcinoma. One (9%) of the mesothelioma cases was positive and its staining score was 1+.

None of the mesothelial proliferation cases were positive for CEA or MOC-31.

When D2-40 and podoplanin staining extent for mesothelioma were compared with each other and compared together with positive mesothelial marker calretinin, no statistically significant staining difference was observed (p>0.05). When CK 5/6 and podoplanin was compared, there was a statistically significant difference. When the mean values were evaluated, it was observed that podoplanin exhibited more staining extent in mesotheliomas (Table II).

The specificity and sensitivity for each positive mesothelial marker were assessed by excluding the mesothelial proliferations and the results were shown in Table III. Specificity for all mesothelial markers was 100% when mesothelial proliferations excluded while it was 77.3% when it was included. The highest sensitivity for mesothelioma was observed with the combination of podoplanin, D2-40 and calretinin and the combination of podoplanin, D2-40, CK 5/6 and calretinin.

Table III: Specificity and sensitivity of positive mesothelial markers in differentiating malignant mesothelioma from primary lung adenocarcinomas

	Specificity	Sensitivity
D2-40	63.6%	100%
Podoplanin	81.8%	100%
Calretinin	81.8%	100%
CK 5/6	54.5%	100%

DISCUSSION

Differentiation of malignant mesotheliomas from lung adenocarcinomas and metastatic carcinomas of the pleura can be very difficult, even impossible, under the light microscope due to the similarity of their histomorphological characteristics. A wide variety of markers have been used in differentiating malignant mesothelioma from primary lung adenocarcinomas (3-9). The differential diagnosis has been based on the principal that the markers that are positive in the cells of lung adenocarcinomas are negative in mesothelial cells. The primary markers used for this purpose are CEA, CD 15 (Leu M1), B72.3, Ber-Ep4, Bg 8, E-cadherin and MOC-31 (2-5,7,10). A variety of markers that are widely synthesized in malignant mesotheliomas but not in primary lung adenocarcinomas have been defined in recent years. Some of these markers are calretinin, CK 5/6, WT-1, mesothelin, HBME-1, OC-125, thrombomodulin, podoplanin and D2-40 (7,10,11).

No specific marker for mesothelioma has been defined yet (7,8,10). Various immunohistochemical panels have therefore been used to differentiate mesothelioma from primary lung adenocarcinomas and other metastatic carcinomas of the pleura. There is a consensus that these immunohistochemical panels should be composed of both positive and negative mesothelioma markers (3,7,8,10,12). Yet, there is no general agreement on a certain immunohistochemical panel (7,8,10).

There have been some recently published studies on the significance of podoplanin, a transmembrane mucoprotein which has been used as an immunohistochemical marker in the diagnosis of mesothelioma (11-13). The results of these studies have shown that podoplanin is often synthesized in epithelioid mesotheliomas but not generally synthesized in sarcomatoid mesotheliomas (11,13). The reported positivity in epithelioid mesotheliomas ranges between 86% and 100% (7); the staining characteristics have been defined to be along the apical surface of the cells and membranous (12,13). Kimura et al. (14) reported that podoplanin was synthesized in all of the 5 epithelioid mesotheliomas but not synthesized in any of the 93 adenocarcinomas of different origins and claimed that this marker can be helpful in differentiating mesotheliomas and adenocarcinomas. In our study, 9 of the 11 (81.8%) mesotheliomas were positive for podoplanin. There was no positivity in other malignancy groups.

Recent studies have revealed that D2-40 can be helpful in differentiating primary lung adenocarcinomas and mesothelioma because it is often synthesized in epithelioid mesotheliomas (8,11,13,15). Chu et al. investigated the significance of D2-40 as a mesothelioma marker in 2005. In their study, a membranous staining was reported in the epithelioid components of 15 (94%) of the 16 biphasic mesotheliomas and in all of the 33 (100%) epithelioid mesotheliomas (13). In 2 studies in which different mesothelioma groups were included, Ordonez reported membranous D2-40 positivity in 37 of 40 (93%) and in 25 of 29 (86%) epithelioid mesotheliomas and argued that D2-40 is one of the most sensitive and specific markers in the diagnosis of epithelioid mesothelioma (11). In our study, 7 of the 11 (63.6%) mesotheliomas were positive for D2-40. No positivity was observed in other malignancy groups.

In parallel with the literature, the results of our study revealed that podoplanin and D2-40 have gained significance in the differential diagnosis of malignant mesothelioma and primary lung adenocarcinomas.

Ordonez et al. (11,15) established in 2 comparative studies that podoplanin exhibits a sensitivity and specificity similar to that of D2-40 in the diagnosis of mesothelioma. Similarly, we observed no statistically significant difference between podoplanin and D2-40 in differentiating malignant mesothelioma and primary lung adenocarcinomas and observed that they have similar sensitivity and specificity.

Calretinin is one of the first positive mesothelioma markers found to be useful in the diagnosis of mesothelioma (7,8). It has been defined as one of the most specific and the most sensitive of the current positive mesothelioma markers (7,8,10) and has therefore been proposed as one of the primary markers to be used in various panels used in the diagnosis of mesothelioma (10). Nine of 11 (81.8%) mesothelioma cases were positive with calretinin in our study. The first study revealing that CK 5/6 might be useful in differentiating lung adenocarcinomas and mesotheliomas was by Blobel et al. in 1985 (16). The reported positive staining was 64-100% in epithelioid mesotheliomas (3,5,17), 0-19% in lung adenocarcinomas (3,5,9,17) and 25-35% in serous carcinomas (8). Six of 11 (54.5%) mesothelioma cases were positive with CK 5/6 in our study. There was no positivity in other malignancy groups.

CEA is the first generally accepted immunohistochemical marker used in differentiating epithelioid mesotheliomas from lung adenocarcinomas (18). Due to its high sensitivity and specificity, CEA is still considered as the best immunohistochemical marker in differentiating these two tumor groups (8). Ten (90.9%) of the primary lung adenocarcinomas and 9 (81.8%) of the bronchioloalveolar carcinoma cases were positive for CEA in our study. But only 1 (9%) mesothelioma was positive with CEA.

Due to its high sensitivity and specificity, researchers have defined MOC 31 as the best negative mesothelioma marker which can be used in differentiating pleural mesotheliomas from both lung adenocarcinomas and lung squamous carcinomas, and peritoneal mesotheliomas from serous carcinomas (7,8). In parallel with the literature, only 1 (9%) mesothelioma case was positive with MOC 31 in our study. On the other hand, 8 (72.7%) of the primary lung adenocarcinomas and 7 (63.6%) of the bronchioloalveolar carcinoma cases were positive.

No certain specific and sensitive marker has been defined for mesotheliomas (2-5). Various immunohistochemical panels including more than one marker have therefore been composed (7,8,10). From a practical aspect, a panel composed of 4 markers, 2 positive and 2 negative mesothelial markers, would allow to differentiate malignant mesothelioma from primary lung adenocarcinomas (2-5,7,8,10).

In our study, when the combination of D2-40 and podoplanin and the combination of D2-40, podoplanin and CK 5/6 were used as positive mesothelioma markers separately, positive results were observed in 10 (90.0%) of the 11 cases. However, when the combination of D2-40, podoplanin and calretinin and the combination of D2-40, podoplanin, CK 5/6 and calretinin were used, all (100%) the cases were stained and the highest sensitivity was obtained.

Our results indicate that CK5/6 can be substituted with D2-40 for improved sensitivity.

When the information obtained from the literature and the results of our study are considered together, it can be concluded that a single marker is not enough to differentiate mesotheliomas from lung adenocarcinomas, bronchioloalveolar carcinomas and metastatic carcinomas of the pleura, and that a panel should be formed with positively and negatively staining mesothelioma markers. In our opinion, a panel formed with podoplanin, D2-40, calretinin and CK 5/6 as positive mesothelial markers together with the negative mesothelial markers CEA and MOC-31 will be more conclusive in problematic cases.

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